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Immunocytochemical localization of APS reductase and bisulfite reductase in three *Desulfovibrio* species

D. R. Kremer[1], M. Veenhuis[2], G. Fauque[3], H. D. Peck Jr.[4], J. LeGall[3,4], J. Lampreia[5], J. J. G. Moura[5], and T. A. Hansen[1]

[1] Department of Microbiology and [2] Laboratory of Electron Microscopy, University of Groningen, Kerklaan 30, NL-9751 NN Haren, The Netherlands
[3] Association pour la Recherche en Bioénergie Solaire, Section Enzymologie et Biochimie Bactérienne, C.E.N. Cadarache, F-13108 St-Paul-Lez-Durance Cédex, France
[4] Department of Biochemistry, School of Chemical Science, University of Georgia, Athens, GA 30602, USA
[5] Centro de Quimica Estrutural, Complexo I, Universidade Nova de Lisboa, P-1096 Lisbon, Portugal

Abstract. The localization of APS reductase and bisulfite reductase in *Desulfovibrio gigas*, *D. vulgaris* Hildenborough and *D. thermophilus* was studied by immunoelectron microscopy. Polyclonal antibodies were raised against the purified enzymes from each strain. Cells fixed with formaldehyde/glutaraldehyde were embedded and ultrathin sections were incubated with antibodies and subsequently labeled with protein A-gold. The bisulfite reductase in all three strains and APS reductase in *D. gigas* and *D. vulgaris* were found in the cytoplasm. The labeling of *D. thermophilus* with APS reductase antibodies resulted in a distribution of gold particles over the cytoplasmic membrane region. The localization of the two enzymes is discussed with respect to the mechanism and energetics of dissimilatory sulfate reduction.

Key words: *Desulfovibrio* – Dissimilatory sulfate reduction – APS reductase – Bisulfite reductase – Enzyme localization – Immunogold labeling

Dissimilatory sulfate reduction is carried out by a large variety of bacteria (Hansen 1988; Widdel 1988). Most studies on its mechanism have been confined to bacteria belonging to the genus *Desulfovibrio* (Peck and LeGall 1982; LeGall and Fauque 1988). Dissimilatory sulfate reduction proceeds as follows:

$$SO_4^{2-} + ATP \xrightarrow[PPi \rightarrow 2Pi]{} APS \xrightarrow[AMP]{2[H]} HSO_3^- \xrightarrow{6[H]} HS^{-1}.$$

Sulfate is activated by ATP-sulfurylase to adenosine phosphosulfate (APS), which is subsequently reduced to sulfite plus AMP by the enzyme APS reductase (LeGall and Fauque 1988). In *D. vulgaris* Hildenborough APS reductase is a non-heme iron flavoprotein containing two different subunits, of 72 kD and 20 kD molecular mass, but it has an unknown subunit structure (Bramlett and Peck 1975). Its natural electron donor is not known. APS reductase has been detected in several genera of sulfate-reducing bacteria (Stille and Trüper 1984).

Offprint requests to: T. A. Hansen

The biochemistry of the reduction of sulfite to sulfide is somewhat controversial. A cyclic route, the so-called trithionate pathway, has been postulated, in which trithionate and thiosulfate are intermediates (Akagi 1981). In this route sulfite is first reduced to trithionate by the enzyme bisulfite reductase. However, strong indications were found that in *Desulfovibrio* spp. this is not the natural pathway (Chambers and Trudinger 1975; LeGall and Fauque 1988) and that in vivo a direct six electron reduction of sulfite to sulfide by the enzyme bisulfite reductase may occur. Whatever route is functioning, bisulfite reductase plays a key role in the dissimilatory reduction of sulfite. Four classes of bisulfite reductases (or dissimilatory sulfite reductases) have been found in different sulfate-reducing bacteria: desulfoviridin, desulforubidin, desulfofuscidin and a P582-type reductase (LeGall and Fauque 1988). All bisulfite reductases thus far examined have an $\alpha_2\beta_2$ subunit structure (LeGall and Fauque 1988). The natural electron donor of bisulfite reductase is not known.

In *Desulfovibrio* spp. electron transport-coupled energy conservation occurs during growth on e. g. lactate, hydrogen or formate in the presence of sulfate (LeGall and Fauque 1988). Several aspects of this process remain to be solved. For an understanding of electron transport-coupled energy transduction it is crucial to know the localization of APS and bisulfite reductases. After cell disruption these non-periplasmic enzymes are generally found in the soluble fraction (Odom and Peck 1981a; Badziong and Thauer 1980) suggesting a cytoplasmic localization. However, the possibility of these reductases being detached form the cytoplasmic membrane during cell disruption cannot be excluded. Since the introduction of postembedding immunogold labeling (Roth et al. 1978) and the low temperature embedding technique in Lowicryl resin (Carlemalm et al. 1982) immunocytochemistry has become a superior method for the localization of proteins. This technique has been successfully used for the localization of bacterial enzymes (Kohring et al. 1985; Ossmer et al. 1986; Aldrich et al. 1987). Ossmer et al. (1986) and Aldrich et al. (1987) showed that in the methanogens *Methanococcus voltae* and *Methanobacterium thermoautotrophicum* Marburg methyl-coenzyme M reductase, an enzyme found in the soluble fraction after cell disruption, occurs in vivo as a membrane-associated enzyme. These results have clearly shown that the

soluble nature of a bacterial enzyme does not necessarily mean that it is a cytoplasmic enzyme. This paper presents an electron microscopical study on the localization of APS reductase and bisulfite reductase with immunochemical techniques in *D. gigas, D. vulgaris* and *D. thermophilus.*

Materials and methods

Organisms and cultivation

Desulfovibrio gigas (NCIB 9332), *Desulfovibrio vulgaris* strain Hildenborough (NCIB 8303), and *Desulfovibrio thermophilus* (DSM 1276) were used in this study. Cells were grown in batch culture in a bicarbonate-buffered medium as described earlier (Kremer and Hansen 1987) containing 20 mM L(+)-lactate, 20 mM sulfate and 0.02% yeast extract (Difco). *D. gigas* was also grown in a pH-controlled (pH = 7.2) continuous culture in the same basal medium (Na_2S was omitted) at a dilution rate of 0.05 h^{-1}, either electron donor-limited (20 mM lactate plus 20 mM sulfate) or sulfate-limited (30 mM lactate plus 10 mM sulfate). *D. gigas* and *D. vulgaris* were grown at 30° C and *D. thermophilus* was grown at 60° C.

Purification of APS reductase and bisulfite reductase

For large scale cultivation the three species were grown on a standard lactate sulfate medium (Starkey 1938) at 37° C for *D. gigas* and *D. vulgaris* and 65° C for *D. thermophilus.* The crude extracts were prepared as previously described (Fauque et al. 1987). The APS reductases from *D. gigas* and *D. vulgaris* were purified after Lampreia et al. (1987). The complete procedure for the purification of APS reductase and desulfofuscidin from *D. thermophilus* will be described elsewhere in detail. A brief description was reported earlier (Fauque et al. 1986a, b). The desulfoviridins from *D. gigas* and *D. vulgaris* were purified as described by Lee and Peck (1971) and Lee et al. (1973), respectively. All enzymes were purified to homogeneity except for APS reductase from *D. vulgaris* which was about 85–90% pure. Purity of the enzymes was established by polyacrylamide disc electrophoresis (Brewer and Ashworth 1969).

Antibody preparation and characterization

Polyclonal antibodies were elicited in rabbits by injecting them 3 times with intervals of 10 days subcutaneously with 0.5 mg purified enzyme in Freunds complete adjuvant (Difco). Serum was treated with 0.25% sodium dextran sulfate and 1% $CaCl_2$ and centrifuged at 16,000 × *g* for 20 min. The supernatant was used as antibody preparation.

Ouchterlony double immunodiffusion was used to demonstrate the presence of antibodies against the purified enzymes.

Immunoblotting was carried out as follows. Cell-free extracts prepared by sonication as described earlier (Kremer and Hansen 1987) were subjected to SDS/PAGE with 10% acrylamide after Laemmli (1970). The protein was blotted onto cellulose nitrate filters by a diffusion blot method (Cohen et al. 1982). The alkaline phosphatase-conjugated antirabbit IgG method (Turner 1986) was used to detect the antigen specificity of the antibodies. A 1000-fold dilution of the antibody preparation was applied. The staining of alkaline phosphatase activity was carried out after Blake et al. (1984). SDS/PAGE of cell-free extracts and purified enzyme stained with Coomassie Brilliant Blue were used to compare the positions of the bands.

Electron microscopy and immunocytochemistry

Cells were fixed in 0.1% formaldehyde plus 0.2% glutaraldehyde for 30 min and subsequently in 1.5% glutaraldehyde for 30 min. Fixation was carried out at 0° C in a buffer containing 100 mM sodium cacodylate pH 7.2, 5 mM $MgCl_2$ and 2 mM $CaCl_2$. Cells were fixed under N_2 immediately after centrifugation under anoxic conditions or washed twice in a 100 mM sodium phosphate buffer pH 7.5 containing 2 mM $MgCl_2$ before fixation. After dehydration in a graded ethanol series and embedding in Lowicryl K_4M at −35° C, polymerization was carried out at −35° C with UV light. Thin sections were cut with a diamond knife and protein A-gold labeling was performed on these sections by the method of Slot and Geuze (1984). Gold particles were prepared by the citrate method of Frens (1973). Immunogold labelings with preimmune sera were used as controls for non-specific labeling.

Freeze-etch replicas of *D. gigas* were prepared by freeze-spraying a suspension of fresh cells without cryoprotectant in liquid Freon followed by freeze-fracturing in a Balzers freeze-etch unit according to the general procedures described by Moor (1964).

Electron micrographs were taken with a Philips EM 300.

Results

Characterization of the antibodies

With the Ouchterlony test all antibody preparations showed one clear precipitation line with their homologous antigen, except for the APS reductase from *D. vulgaris* which gave two precipitation lines. No cross-reactivity occurred between antibodies against the two enzymes from *D. thermophilus* and cell-free extracts from the other *Desulfovibrio* strains. Some cross-reactivity occurred between antibody preparations against the two enzymes from *D. gigas* and cell-free extracts from *D. vulgaris* and vice versa (Fig. 1). None of the preimmune sera gave precipitation lines with the purified enzymes or with the cell-free extracts (not shown).

Immunoblotting showed that antibodies were raised against both subunits of APS reductase from *D. gigas, D. vulgaris* and *D. thermophilus* and bisulfite reductase from *D. gigas.* Only the 41 kD subunit of *D. vulgaris* bisulfite reductase, however, reacted with the antibodies at the dilutions used. No other bands were stained than those which coincided with the SDS/PAGE bands of the purified enzymes (Fig. 2).

The antibody preparations against APS reductase from *D. gigas* and *D. vulgaris* showed a small tendency to attach to the cell wall and the fimbriae of the respective organisms, as was revealed by the immunogold-labeling technique; 10% to 20% of the gold particles were present at these sites (not shown). Possibly a small contamination was present in the purified enzymes which could not be shown during immunoblotting but was highly antigenic. The contaminating antibody fraction could easily be removed by adding a suspension of freshly grown washed cells of the particular strain to the antibody preparation (50 μg cell protein/ml). After careful mixing, the cells were removed by centrifuga-

tion. The supernatant of these antibody preparations still had a comparable activity against the purified enzymes when examined with the Ouchterlony test (not shown). The two preparations thus obtained were used for further studies on the localization of the APS reductases in *D. gigas* and *D. vulgaris*.

Immunochemistry

The results of the immunogold-labeling experiments of the APS reductases and the bisulfite reductases in the three strains are shown in Fig. 3. Gold-labeling of APS reductase in *D. gigas* and *D. vulgaris* and bisulfite reductase in all three strains resulted in a scattering of gold particles over the cytoplasm. Labeling of APS reductase from *D. thermophilus* led to a clear distribution of gold particles in the cytoplasmic membrane region. Specific labeling was absent when preimmune sera were used in these experiments. No differences in the pattern of labeling could be seen when a different fixation procedure was used.

Because enzyme localizations may be dependent on the growth phase of the organisms (Rohde et al. 1985), cells were taken from the early-, mid-, and late exponential phase (OD_{660} = 0.050, 0.20 and 0.45) and cells of *D. gigas* were also taken from lactate-limited and sulfate-limited continuous cultures. No differences could be seen in the localization of the two reductases in the variously-grown strains. However, morphological differences resulting form the various growth conditions became evident. In cells of all strains from the late exponential phase and cells of *D. gigas* from the sulfate-limited continuous culture, large amounts of granules with low electron density were present, presumably polyglucose (see Stams et al. 1983), whereas cells from the early exponential phase contained little and *D. gigas* from the lactate-limited continuous culture contained hardly any polyglucose. These granules were not labeled (Fig. 3B, C). The low electron dense DNA region in the centre of the cells was not labeled either (Fig. 3A). The massive accumulation of polyglucose (and the presence of DNA) in the centre of the cell may have caused a somewhat higher labeling in the periphery of sulfate-limited cells of *D. gigas* (Fig. 3C). An analysis of more than 20 sections at different magnifications however showed that by far the major part of the labeling was present in the cytoplasm. *D. gigas* contained internal membrane structures especially in the early exponential growth phase and when grown in continuous culture. These membrane structures and the sites of the gold-labeling of the two enzymes did not coincide to any significant extent

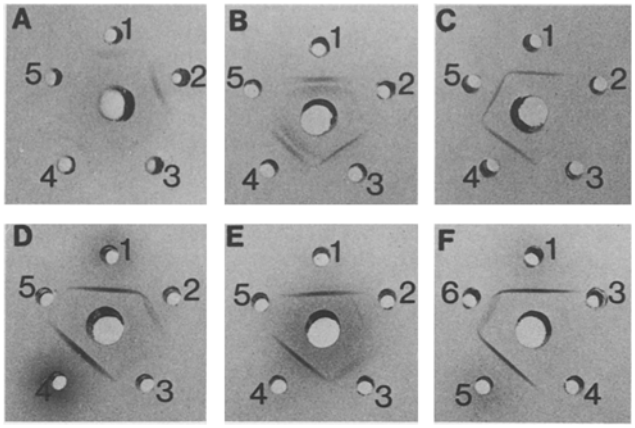

Fig. 1A–F. Ouchterlony double immunodiffusion tests. **A–C** Demonstration of the presence of antibodies against the APS reductases from *Desulfovibrio gigas, D. vulgaris* and *D. thermophilus*, respectively. **D–F** Demonstration of the presence of antibodies against the bisulfite reductases from *D. gigas, D. vulgaris* and *D. thermophilus*, respectively. *Well 1* and *4* contain the purified enzymes. The central wells contain the antibody preparations against the purified enzymes in *well 1* and *4*. *Well 2, 3* and *5* contain cell-free extracts form *D. gigas, D. vulgaris* and *D. thermophilus*, respectively

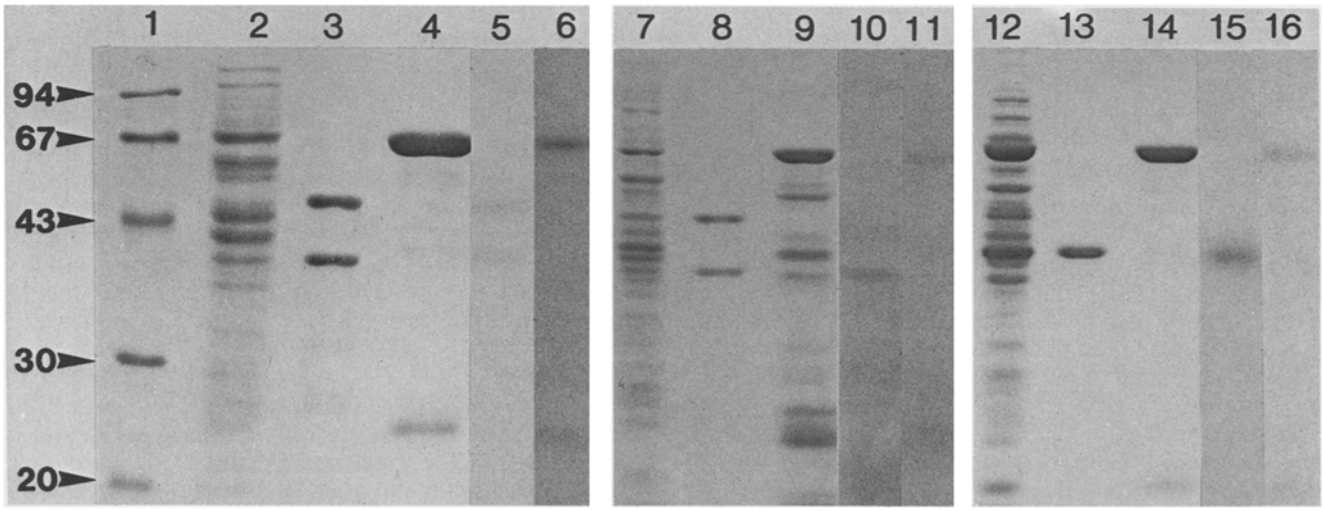

Fig. 2. Characterization of the antibody preparations with immunoblotting. *Lane 1*: SDS/PAGE of the following molecular markers: phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor. Molecular masses are given in kD. *Lanes 2–6:* characterization of the antibody preparations against the two reductases from *D. gigas*; *lanes 2–4:* SDS/PAGE of the cell-free extract, the bisulfite reductase and APS reductase from *D. gigas*, respectively; *lane 5:* blotted cell-free extract from *D. gigas* labeled with antibodies against the bisulfite reductase from *D. gigas* stained with the alkaline phosphatase-conjugated goat antirabbit IgG method; *lane 6:* blotted cell-free extract from *D. gigas* labeled with antibodies against the APS reductase from *D. gigas* stained with the alkaline phosphatase-conjugated goat antirabbit IgG method. The characterization of the antibodies against the two reductases from *D. vulgaris* (*lanes 7–11*) and from *D. thermophilus* (*lanes 12–16*) was carried out as described for *D. gigas*

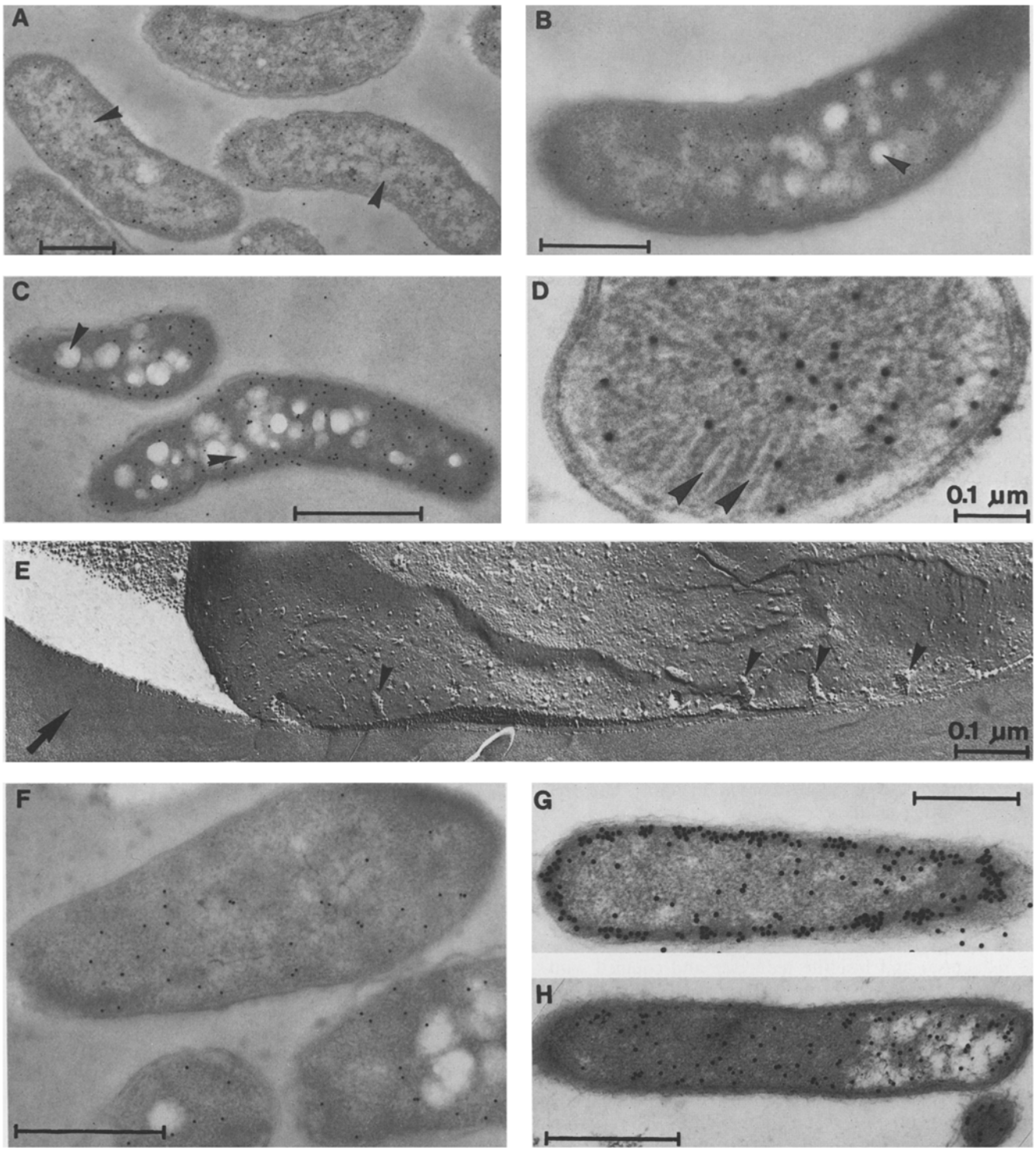

Fig. 3A–H. Immunogold labeling of APS reductase (**A, C, D**) and bisulfite reductase (**B**) in *D. gigas*. Cells from the mid exponential phase were used in **A, B**. Sulfate-limited and lactate-limited continuous culture cells were used in **C, D**, respectively. Note that the DNA regions (arrows in **A**) and the polyglucose (*arrows* in **B, C**) are not labeled. The membrane structures in **D** (*arrows*) could also be shown in freeze-etch replicas from early exponential cells of *D. gigas* (**E**, *arrows*). Immunogold labeling of APS reductase in cells from the mid exponential phase of *D. vulgaris* is shown in **F**. Labeling of bisulfite reductase in this organism gave comparable results (not shown). Labeling of APS reductase and bisulfite reductase in cells from the early exponential phase of *D. thermophilus* is shown in **G, H**, respectively. Gold particles of 10 nm (**A–D, F**) or 20 nm (**G, H**) were used. Unless otherwise indicated, bar = 0.5 µm

(Fig. 3D). The membrane structures in *D. gigas* appeared to be invaginations of the cytoplasmic membrane, as shown by the freeze-etch technique (Fig. 3E).

Discussion

The bisulfite reductases in all strains and the APS reductases in *Desulfovibrio gigas and D. vulgaris* appear to be localized in the cytoplasm. In the case of CO dehydrogenase of *Pseudomonas carboxydovorans* and methyl-coenzyme M reductase of *Methanobacterium thermoautotrophicum* Marburg, which are functionally present at the cytoplasmic membrane, a large fraction of the enzymes was found in the cytoplasm under some growth conditions, possibly due to over-production of the enzymes (see Ossmer et al. 1986; Aldrich et al. 1987; Rohde et al. 1985). Under none of the growth conditions any association of APS reductase in *D. gigas* and *D. vulgaris* and the bisulfite reductases in all strains with the cytoplasmic membrane was observed. If these enzymes would be functionally membrane-associated, one would expect at least under some conditions a substantial amount of the gold label to be localized at the cytoplasmic membrane. APS reductase in *D. thermophilus*, however, was clearly membrane-associated under all growth conditions tested.

The difference in the subcellular localization of APS reductase between *D. thermophilus* and the two other strains was surprising. Apart from its thermophilic growth, *D. thermophilus* seems related to the other *Desulfovibrio* strains with respect to the presence of some typical *Desulfovibrio* redox carriers (Fauque et al. 1986a). Most likely APS reductase in *D. thermophilus* accepts electrons directly from a membrane localized electron transport chain, a situation analogous to O_2 respiration, nitrate respiration, fumarate reduction and possibly methyl-CoM reduction (Thauer et al. 1977; Aldrich et al. 1987). There are other examples of membrane-associated APS reductases. In purple sulfur bacteria belonging to the Chromatiaceae, where APS reductase catalyzes the oxidation of bisulfite + AMP to APS, the enzyme is associated with the chromatophores (Trüper and Fischer 1982).

The mechanism of electron transfer to the cytoplasmic APS reductases and bisulfite reductase and coupled with this the mechanism of electron transport-mediated energy conservation remain to be elucidated. During growth on hydrogen or formate electrons are released in the periplasm and therefore have to be transferred via membrane-associated redox carriers across the membrane. During growth on lactate the reducing equivalents from the pyruvate/lactate couple most likely enter a membrane-localized electron transport chain, because L-lactate dehydrogenase is a membrane-bound enzyme (Stams and Hansen 1982). This means that (at least part of) the reducing power must be carried from a membrane-localized electron transport chain to APS reductase and bisulfite reductase in the cytoplasm. In *D. gigas* flavodoxin and ferredoxin II are possible candidates as natural electron donors of bisulfite reductase (Barton et al. 1972; Moura et al. 1978). It would be interesting to see if flavodoxin and ferredoxin II can accept electrons from a membrane-bound redox carrier. Knowledge of the mechanism of transfer of reducing equivalents to APS reductase and bisulfite reductase will add to the refinement of models that have been proposed for electron transport-mediated energy conservation in *Desulfovibrio* (Badziong and Thauer 1980; Odom and Peck 1981b; Lupton et al. 1984).

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